to evaluate the monomeric nature of mPapaya1, the protein was evaluated using the recent protocol of Costantini et al. (2012). In this assay, a test protein (mPapaya1, for example) is fused to the cytoplasmic end of the endoplasmic reticulum (ER) membrane protein CytERM. If the fusion pair homo-oligomerizes, the structure of the ER is changed from a tubular network into a visible network of organized smooth ER whorls. Thus, counting the percentage of cells with whorls indicates the level of oligomerization of the test protein. In this study, 83% of cells with the mPapaya1 fusion had a tubular ER morphology, compared to 77% of cells expressing the monomeric control protein mEGFP, suggesting that mPapaya1 is essentially monomeric. As further support for its monomeric nature, mPapaya1 was fused to multiple partners (histone H2B, connexins, tubulin, and others) and observed with microscopy, and the fusion was seen to not interfere with the normal trafficking or localization of these proteins.

As a final test, the authors evaluated mPapaya1 as a partner for FRET. This was important, because one of the motivations to create mPapaya1 was to find an alternative yellow FRET acceptor. To test the response of mPapaya1 in a FRET assay, the authors created a proteolytically-sensitive mPapaya/mTFP1 fusion. This donor/acceptor pair should have a Förster radius of 5.1 nm. This fusion showed a strong FRET signal and a large decrease in FRET upon activation of the protease both in vitro and in vivo. Thus, mPapaya1 should act as an excellent yellow FRET acceptor for structural studies or the creation of new biosensors.

Completely rational protein design has seen limited success. Perhaps this is the result of the complex and interconnected nature of a protein's structure and function (Fleishman and Baker, 2012). Often, non-intuitive mutations are needed to improve the folding, activity, or stability of a protein. A major boon for FP design is the ability to rapidly screen for function—namely, color and intensity. Similar screening methods to look for other features in proteins, such as enzyme activity, ligand binding, or optical qualities, are also being combined with massive directed evolution methods to produce new engineered proteins with optimized behaviors (Fleishman and Baker, 2012; Romero and Arnold, 2009). In addition to providing an exciting new FP, the work presented here demonstrates the ability of directed evolution to fill in gaps in the collection of nature's protein library.

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Finding Epitopes with Computers

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The goal of structural vaccinology is to enable the design and engineering of improved antigens. In a recent issue of *Chemistry & Biology*, Gourlay and colleagues provided evidence that structure-based computational methods allow prediction of B cell epitopes, a crucial step for antigen selection and optimization in vaccine development.

Understanding the molecular bases of immune recognition is one of the big challenges in vaccine development, and the elucidation of the three-dimensional (3D) structure of antigens and antigen-antibody complexes is central to structural vaccinology. This provides knowledge on which structural and dynamic properties might be responsible for the

ability of antigens to elicit functional or protective antibodies (Dormitzer et al., 2008, Kulp and Schief, 2013). Although genomic and proteomic methods, now widely utilized, provide key information on secreted or surface-exposed vaccine target candidates, labor-intensive research is still required for the selection of the best vaccine antigens (Sette and Rappuoli, 2010). This is mostly due to the necessity of elucidating the biochemical and immunological properties of each single antigen experimentally, which is essential in order to formulate vaccines using the most stable and immunogenic antigen configurations or conformations (i.e., stable molecules that are easily recognized by the immune system and thus able to elicit an appropriate immune response). One of the pivotal activities in this pipeline is the discovery of those regions of the antigen that are involved in forming the interface with the antibody.

So far, understanding the properties that control the interactions between antigen epitopes and antibody paratopes requires atomic-level information of their complexes. To date, over 200 nonsimilar antigen-antibody complex structures have been deposited in the protein data bank (PDB), providing a database from which to extract conserved features of such interactions. Several research teams have attempted to define a series of rules devised to explain the desirable properties of amino acids usually forming an epitope (Kringelum et al., 2013), like the complementarity of epitope-paratope surfaces (Greenspan and Cooper, 1995), the affinity and specificity of their interaction (Padlan et al., 1995), or the extent to which they are exposed on the surface (Novotný et al., 1986). In cases where it is not possible to elucidate the full 3D structure of an antigen-antibody complex, which is a rather time consuming activity, methods that allow the reliable and quick identification of putative epitopes are needed. If the 3D structure of the antigen alone is available, it is now possible to use its atomic coordinates for the prediction of immunogenic regions, which can then be used for generating optimized antigens in the form of synthetic peptide epitopes.

In the September issue of *Chemistry & Biology*, Gourlay et al. (2013) showed that computational epitope predictions, performed with two independent in silico methods, were able to elucidate the presence of an antigenic epitope, which was then confirmed to elicit bactericidal antibodies when presented as a synthetic peptide. From the crystal structure of the acute phase antigen Pal-Bp (BPSL2765) of *Burkholderia pseudomallei*, originally identified by reverse vaccinology, the authors used molecular dynamics simulations to select the most representative ensemble of structures, which were successively employed for epitope prediction. This was performed by analyzing separately physicochemical properties of the antigen surface, such as its solvation, energetics, and topology. Specifically, the authors used the electrostatic desolvation profiles (EDP) (Fiorucci and Zacharias, 2010) and the matrix of local coupling energies (MLCE) methods (Scarabelli et al., 2010). Experimental epitope mapping using polyclonal antibodies was then performed by partial digestion, immunocapture, and mass spectrometry, and consensus models between the epitopes found by computational and experimental methods were built. The resulting consensus regions of Pal-Bp were synthesized, and their immunoreactivity to human plasma antibodies, neutrophil opsonization killing, and bacterial agglutination activities were investigated.

A single highly immunogenic region of Pal-Bp (EPITOPE 3) was found, and antibodies raised against a synthetic epitope generated around this epitope were significantly more active (as tested by opsonization killing using human neutrophils and in assays of antibody-mediated *B. pseudomallei* agglutination) when compared with those generated against the full-length recombinant protein. The authors hypothesize that this enhanced immunogenicity could originate from a smaller recognition sequence or from a more efficient exposure of the peptide epitope than in the full-length conformation. Importantly, the dissection of fully folded antigens in subdomains was previously explored by the same authors (Genoni et al., 2012; Lassaux et al., 2013) in order to overcome the main limitation of the EDP and MLCE methods that use the full-length 3D folded structures to analyze their surface physicochemical properties. Clearly, processing an antigen might expose regions that, in the fully folded protein, are not necessarily exposed or available for recognition by antibodies.

This work confirms how a structural understanding of the molecules involved in immune recognition can guide vaccine development (Dormitzer et al., 2012) and how structure-based computational methods are now becoming valuable tools for extending the scope of structural vaccinology. By building on prior knowledge of the atomic and molecular bases of antigen-antibody recognition, these new computational methods seem poised to provide the tools needed for the design of more efficacious vaccines. For example, the generation of libraries or databases of potential epitopes available for synthesis would revolutionize the way antigens are presently screened and selected for vaccine development.

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